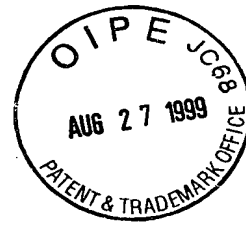


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pertaining to a

Test procedure with biological system

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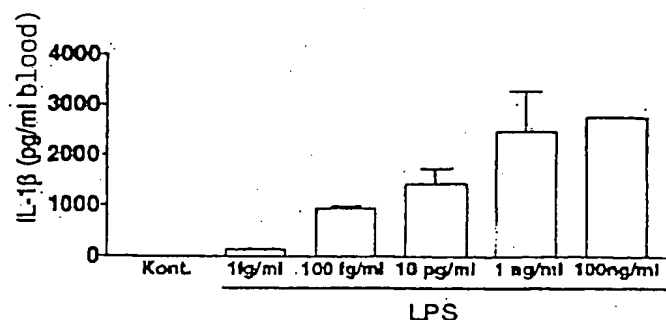
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The following information has been excerpted from the documents filled by the applicant

54 Test procedure with biological system

57 Preparations containing deep-frozen
blood are used for test procedures for
determining blood response

IL-1 β release in LPS - stimulated,
deep-frozen 10% whole blood



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Description

The invention is in the field of use of biological systems for tests. Biological systems have long been used to a substantial extent for tests. In these tests the test substances, i.e., the object, material, or device being tested is brought into contact with biological system. Then the response (which may include non-response) of the system is detected qualitatively and/or quantitatively and evaluated. Such procedures are quite common in dermatology. There, the skin of test subjects, or skin preparations, are used as a biological system to carry out such biological tests, and the response of the skin, or skin preparation in contact with a test material is determined. Incubated chicken eggs are used for tests for mucous membrane irritation. Slaughterhouse products of all types are used as biological systems for a wide range of tests.

This invention concerns the special area of blood as a biological test system. For example, it is known that many materials, on contact with human tissues, body fluids and cells, can activate or, in harmful form, overactivate, the cells of the immune system. Sometimes the response is because the materials themselves stimulate the immune system. At other times, it is because the samples contain immune-stimulating components such as pyrogens, which cause fever. The materials with such a hazard potential include medications in particular, and especially products which can be inhaled, injected, and infused. They include blood substitutes and blood replacements, plastics of various states of aggregation, shapes, and uses, including dispersions and materials such as membranes and prostheses, as well as materials isolated from natural products including plants, animals and humans, or their cell or tissue cultures, isolated products such as vaccines, medications, and genetic therapeutic agents. Materials which cause release of factors such as cytokines from the leukocytes of the organism when in contact with human tissue, cells, or body fluids are called immunoactivators. More narrowly, they are called pyrogens if the factors released can cause a fever reaction in the organism. Product safety is a pressing requirement in pharmaceuticals; therefore, routine individual tests on experimental animals are needed to recognize exceptional contaminated lots. Instead of the pyrogen test on rabbits, as is specified in various pharmacopeias, or the Limulus amoebocyte lysate (LAL) test, which, however, detects only some of the pyrogens and immunoactivators, namely, the endotoxins of gram-negative bacteria, the same inventors have therefore previously described a simple, economical, and widely applicable procedure (European Patent 0 741 294 A2, Hartung and Wendel, ALTEX 12, pp. 70-75,

1995) in which blood or blood preparations, as the biological system, are brought into contact with the material to be tested and the release of cytokines, e.g., leukocyte factors such as interleukin-1 ($IL-1\beta$), which produce fever in the organism (endogenous pyrogens) is measured. It is not unusual for the blood response to be needed as soon as possible, and this applies generally in cases where whole blood is required as a biological system for the test. In such cases the test of the test material must be started without delay, and in any case, within a few hours. That leaves no time for thorough checking of the reactivity of the whole blood used, and it is impossible to rule out with the necessary reliability the possibility that the results might be incorrect, with dangerous consequences, or unusable, due to use of blood from a human donor with abnormal reactions such as might be caused by genetic variations, disease, or life style. As in the case of testing of individual lots of pharmaceuticals, mentioned as an example, the danger of improper transportation or storage often requires that the materials be tested before use. But, for the reasons noted above, when blood is used as a biological system, it may not be possible to make a comparison with previous test results with sufficient reliability.

One of the objects of the invention is, accordingly, to ensure a smooth and reproducible procedure, ruling out the possibility of error due to abnormal reactions, and to provide a method which allows test results with the same test material to be compared when blood is used as the biological system for testing. It is intended that repeated tests at various sites be standardized and made possible at various times. Other objectives arise from the advantages pointed out below.

The objective is established by use of deep-frozen blood or of a preparation containing deep-frozen blood for biological test procedures for, or with, determination of the blood response on contact with the test material.

Use of deep-frozen blood or a preparation containing such blood makes it possible to have blood which has been previously tested and is thus known to be free of abnormal reactions available as a biological system at any time. At the minimum it can be standardized, and then used as a standard reagent. As a large number of completely identical deep-frozen units can be prepared from one lot of blood, the requirement of test reproducibility at different times and places can be satisfied. Furthermore, characterization

of one such lot with data relevant to the testing provides a means of comparison with test results from different lots of blood which have also been adequately characterized.

Whole human or animal blood, from which no components have been separated, has proven to be good as the deep-frozen blood. Freshly drawn blood from human donors is found to be particularly good. The individual components, including the leukocytes, are present in their natural environment, with all the serum components that might influence the action of the test material. Use of deep-frozen whole blood is, therefore, greatly preferred, even though use of leukocytes isolated from the blood is not ruled out. However, most methods of isolation are very expensive for routine testing, so that the use of whole blood is simpler, more reliable for the desired result, and more informative because of the possibility of interactions among the other blood components.

In any case, the deep-frozen product must be thawed for the biological test procedure. The test material is brought in contact with the blood, or with preparations thereof, for a period of such length and in a manner such as is required to produce an adequate blood response. Contact times of some seconds or minutes can be sufficient, without ruling out contact times of several hours' duration. Any contact form suitable for achieving the specified purpose can be used. Examples include immersion, flushing, or draining for objects, and mixing with, or adding to the blood or blood preparations and the like for solutions, dispersions, cultures and solids. The usual biological, physical, chemical and/or physicochemical methods of determination and measurement can be used to determine the blood response routinely after even brief incubation of the blood or blood preparation following contact with the test material. These include biological assays and RIA procedures, preferably spectrometric procedures, all procedures which provide qualitative and quantitative measurement of materials released or formed, such as ELISA, or turbidimetric or chemiluminescent procedures, etc.

The preparations with deep-frozen blood, or leukocytes, can contain diluents, components to delay clotting, cryopreservatives, and other additives which are themselves known, as long as they do not affect the test result. Isotonic solutions such as isotonic sodium chloride solution, Ringer's solution and cell culture media such as RMPI 1640 have proved good as diluents. It is advantageous not to add the diluent until the test is performed, namely during or after thawing, eg, at 37°C. The diluents may, for example,

make up 5 to 95% by volume of the total preparation. Dilutions of 1 to 10 parts by volume are often used.

The deep-frozen blood reagent can be produced by mixing the blood or blood preparation with cryopreservatives, dispensing the mixture, if necessary, in appropriate portions, and then deep-freezing it. Examples of suitable cryopreservatives include organic solutions, inorganic, even salt-containing solutions, or mixtures thereof in variable proportions. For instance, it has proved particularly suitable to use 10 % dimethylsulfoxide alone, or glycol or glycerin alone or mixed with dimethylsulfoxide. Controlled slow freezing at, for example, -1°C per minute is advantageous. A storage temperature of -70°C in an upright deep-freezer has proved good for the frozen blood, as well as use of condensed or solid gases such as liquid nitrogen or dry ice. The latter is particularly suitable for transport.

The deep-frozen preparation can contain clot-delaying components, or they may be added during or after thawing or while the test is being carried out, for example, during an incubation.

Examples of suitable clotting inhibitors include sodium citrate at, for instance, a final concentration of 0.38 % by weight, or heparin, such as sodium heparin, heparin fractions, and the like.

The use according to the invention of preparations containing deep-frozen blood, including deep-frozen blood or deep-frozen leukocytes, is of particular advantage for the testing of materials such as the objects, materials, and devices mentioned initially for immunostimulatory or immunomodulatory effects, or for the qualitative and/or quantitative determination of immune-related effects, through discovery, determination, or evaluation of the blood response on contact of these test materials with the thawed deep-frozen blood or blood preparation.

Besides having immunostimulatory effects, such as pyrogenicity, materials can modulate the subsequent activation of the immune system. This may be desirable for immunotherapeutic agents, but is often an undesired toxic effect. An effect of this kind should be sought after in the development of immunopharmacologic agents, but should be ruled out for many other materials.

Of therapeutic interest are, on the one hand, immunostimulants for the treatment of absolute or relative immune deficiency, and, on the other hand, immunosuppressive agents and antiinflammatory agents, including antiphlogistics, antirheumatics, and antiallergics. The immunotoxic effects of materials, such as substances, have attracted increasing public attention in recent years. There is, as yet, no standardized test system for immunopharmacologic or immunotoxologic materials testing. Stimulated whole blood, though, is finding increased scientific use for characterizing the pharmacologic properties of active substances [Hartung et al., Blood 85 (1995) 2482-2489].

Standardization of the blood used is the central problem connected with the use of blood and its factor release for the testing of immune-related effects, such as the determination of pyrogens, immunomodulators and immunotoxins.

Here, too, the aforementioned blood preparations can be used, such as whole blood, perhaps diluted with, for example, cell culture media, buffers, or clinical sodium chloride solution. In these cases, it is very often necessary to start the test within a few hours, so that it is generally impossible to carry out an advance suitability test on the blood material used. The objective previously stated is intended to allow testing of materials for immune-related effects such as pyrogenicity, immunomodulatory or immunotoxic effects, and should make it possible both to test the blood in advance and to test a material repeatedly at various times and places with the same test blood. That is reliably accomplished by the application according to the invention.

The particular advantage of the invention consists in the determination of immune-related effects and data when use is made of a biological system providing information relevant to human exposure. The measured parameters include cytokines such as the endogenous pyrogens interleukin-1 β , interleukin-6 and tumor necrosis factor; eicosanoids such as the endogenous pyrogen prostaglandin E₂, or other substances released from leukocytes, such as degranulation products, soluble receptors, proteins, or low-molecular-weight substances (see also European Patent O 741 294 A2, to which reference is made here). It is noteworthy that in this procedure not only the clotting inhibitors citrate and heparin have no influence that would falsify the result, but in addition, that the deep-freezing preservatives, including the cryopreservatives mentioned, yield a blood which, after thawing, exhibits no disadvantageous effects on the test result.

The invention has a whole series of other advantages. After thawing of the deep-frozen blood it is again possible to detect inherent physiological reactions of native immune cells under natural conditions and mixing ratios.

Due to deep-frozen preservation it is possible to use the same blood repeatedly at different times and places. Abnormal blood reactions can be recognized in appropriate preliminary tests and the corresponding material can be removed. Standard values for the particular lot of blood can be determined in advance under standardized conditions. It makes it possible to conduct tests even if it is not immediately possible to draw blood. It is possible to determine not only, for instance, direct immunoactivators but also immunomodulators/immunotoxins through their modulation of the effect of a standard stimulus such as endotoxin (lipopolysaccharides of gram-negative bacteria). The invention is also suitable for testing pharmacological therapies or exposures *ex vivo*. In this case, blood is taken from the corresponding patients, test persons, or test animals after administration of the pharmaceutical. It is deep-frozen in the due manner, or cryopreserved, and is only tested later for, as an example, reactivity to a standard stimulus.

Another object of the invention is a preparation containing a collective, containing or consisting of deep-frozen blood in the form of standardized unit doses for biological test procedures which involve determination of the blood response on contact with test material.

It is preferable to use fresh whole blood from healthy animals or human donors. It may, if desired, be mixed with cryopreservatives, diluents, and/or clotting inhibitors, as previously described by way of example, metered out in portions and deep-frozen. The unit dose usually contains from 50 to 500 microliters, preferably 100 microliters, of whole blood, but it is not limited to these quantities. To perform the test procedure, one unit dose of deep-frozen blood is thawed. It is made up, for example, to a desired volume, such as 1 milliliter, with diluents, such as those of the type previously mentioned, and brought into contact with the test material. The blood response is determined in the usual manner and is evaluated. The volume of whole blood that can be drawn at one time from a healthy human donor allows preparation of several thousand unit doses of, for example, 100

microliters. The unit doses can be combined in sets or collectives made up of, for instance, 5, 10, or more unit doses. If desired, data usable for the test procedure can be provided. They can be distributed commercially in deep-frozen form. The set can, for specific applications, contain not only units with unit doses but also units with a multiple of the unit dose.

The particular unit dose can, by the way, contain the same components and have the same composition, and be prepared and used in the same manner, as the previously described preparations containing deep-frozen blood.

Example

Citrated blood from healthy donors was mixed with 10% dimethylsulfoxide (Merck, Darmstadt) immediately after it was drawn. Aliquots of 100 microliters were distributed in 2 milliliter reaction vessels (Eppendorf, Hamburg) and transferred in a commercial freezing system (Mr. Freezy, Nalgene) to a -70°C freezer. The blood was thawed in a thermoshaker (Eppendorf, Hamburg) at 37°C . Immediately after thawing, 900 microliters of RPMI 1640 (Gibco, Eggenstein), warmed to 37°C , were added. Then the test substances, such as dilutions of a pyrogen, in this case, lipopolysaccharide of *Salmonella abortus equi* (Sigma, Deisenhofen) were added. After a four-hour incubation at 37°C , for example, in an incubator (Heraeus, Fellbach) with 5 % carbon dioxide the incubated samples are shaken and centrifuged. Endogenous pyrogens, IL- 1β in this case, are determined in the cell-free supernatant, after freezing, if desired. Figure 1 shows the formation of IL- 1β in the cryopreserved blood from four healthy donors as a function of the amount of pyrogen added. IL- 1β was determined by ELISA (enzyme-linked immunosorbent assay) from antibodies from the company Pharmingen (Hamburg). The test shows release of endogenous pyrogens in the presence of minimal amounts of pyrogens.

Figures 2 and 3 show release of IL-1 in frozen whole blood stimulated with LPS as a function of the amounts of azathioprin or dexamethasone administered.

Claims

1. Method of using deep-frozen human or animal blood, or a preparation containing deep-frozen blood, for biological test procedures involving determination of the blood response to contact with test material.
2. Method according to Claim 1, in which a deep-frozen preparation containing leukocytes isolated from blood is used as blood.
3. Method according to Claim 1 or 2, in which the preparation contains cryo-preservative agents.
4. Method according to one of the preceding claims, in which the preparation contains clotting inhibitors.
5. Method according to one of the preceding claims for determining immune-related data.
6. Collective preparations for biological test procedures involving determination of the blood response to contact with test material, comprising units which each contain deep-frozen blood in the form of a standardized blood unit dose.
7. Collective according to Claim 6, containing, in addition to units which each contain a standard unit dose, units which each contain a multiple of the unit dose.
8. Collective according to Claim 6 or 7, in which the preparations contain cryopreservative agents, clotting inhibitors, and/or diluents.
9. Collective according to one of the preceding claims for determining immune-related data.

In addition, 2 pages of drawings

Fig. 1

IL-1 β release in LPS - stimulated deep-frozen 10 %
whole blood

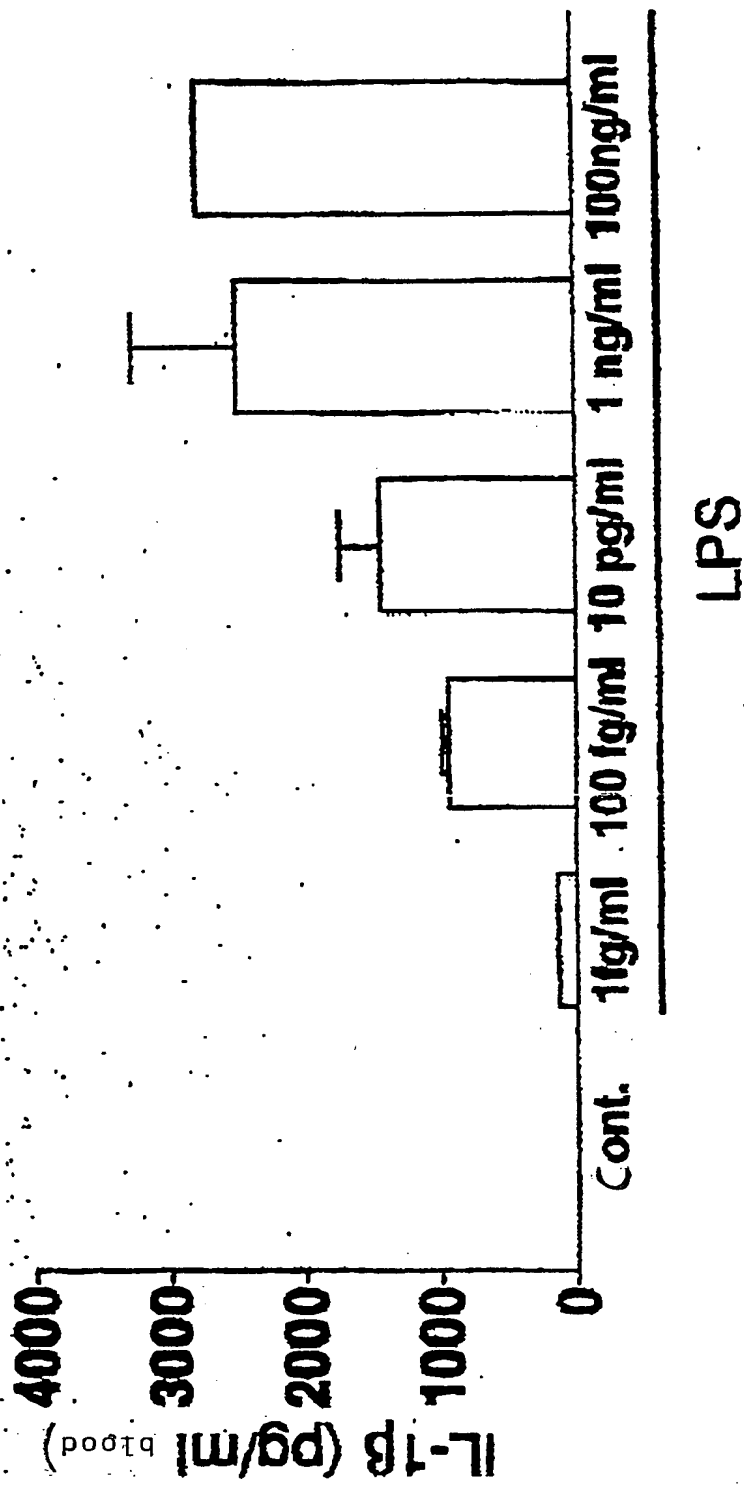


Fig. 2:

IL-1 release in LPS - stimulated,
deep-frozen 10 % whole human blood

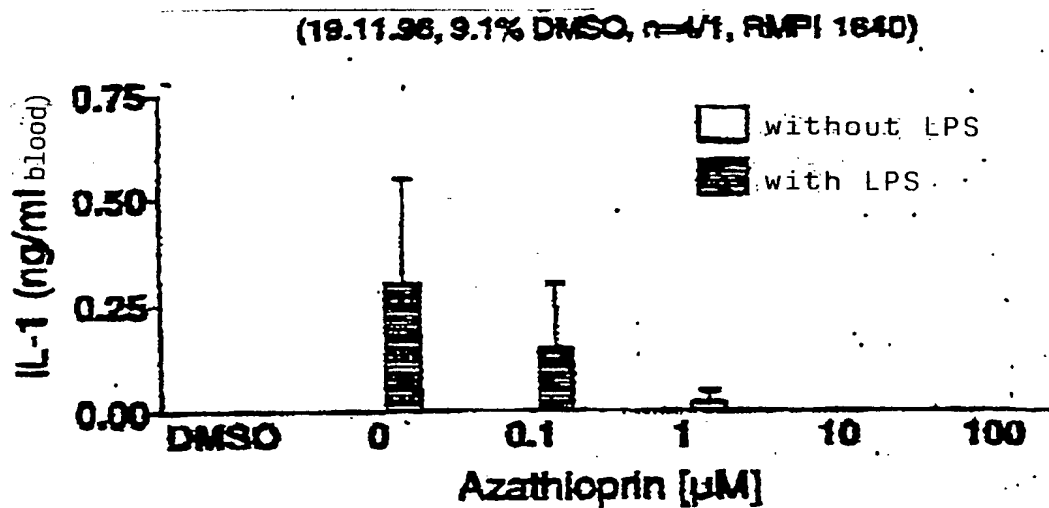
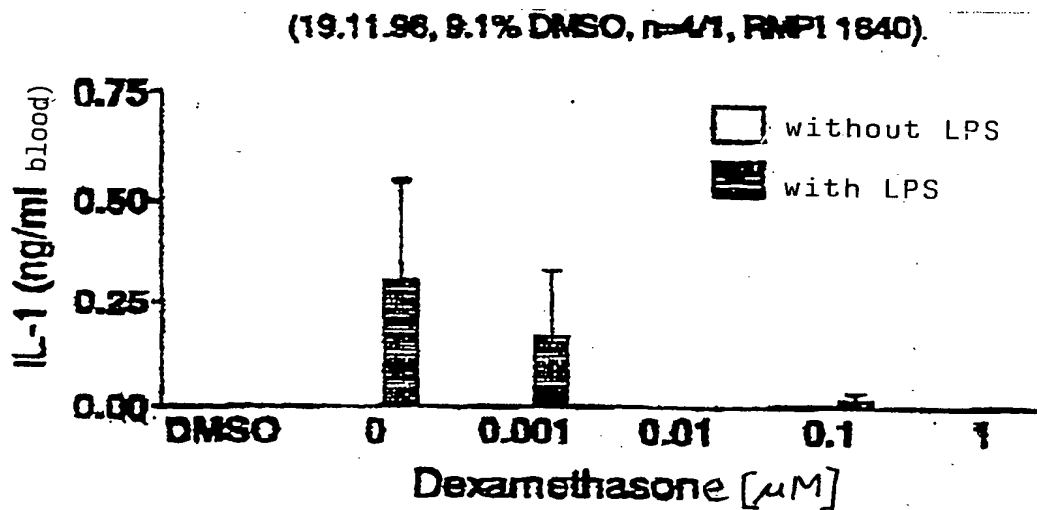


Fig. 3

IL-1 release in LPS - stimulated,
deep-frozen 10 % whole human blood



*In my capacity of public translator for the German and English languages, duly commissioned and sworn in by the President of the Regional Court of Ingolstadt in the Federal Republic of Germany, I hereby verify that:
the translation hereinbefore of the German-language laid-open patent application No. 196 54 266 A 1 submitted to me is exact and complete.*

Steinkirchen, 22nd July, 1999

Jennifer Pockrandt

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